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STAIN TECHNOLOGY

APRIL, 1927

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STAIN TECHNOLOGY

VOLUME II APRIL, 1927 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

STUDIES OF PURE DYE COMPOUNDS

One of the chief obstacles in the standardization of stains has been the difficulty in getting pure dye compounds in order to learn just what dye is needed for some special purpose. A most striking illustration of the point is the matter of gentian violet, a dye which is well known to be a variable mixture, as has been frequently discussed in these pages and in reports of the Commission before this journal was started.

The presence of foreign ingredients, however, such as sodium chloride or dextrin (which in the past have sometimes made up as much as 50% of the total bulk) is one of the least important sources of error in such work. Much more difficult to contend with is the fact that the stains, as ordinarily marketed, contain mixtures of various dye compounds. Thus gentian violet is known to have been a mixture of at least three different methylated pararosanilins and possibly of some other dyes also. Basic fuchsin generally contains two and may contain four different dyes of the group known as fuchsins. Acid fuchsin is a sulphonated derivative of basic fuchsin and may vary greatly in the degree of sulphonation as well as in the composition of the basic fuchsin from which it is derived; as a matter of fact, there are twelve different pure dye compounds theoretically possible in an acid fuchsin.

The dyes marketed are ordinarily designated in some way to indicate their shade; and as shade is related to chemical composition, the shade designation often gives some idea as to which of the various possible compounds predominates. Such information is very indefinite, however, and does not furnish a satisfactory basis for work of real scientific value.

This fact caused a decided handicap in the early investigations on stains. In the first comparative work on gentian violet, for instance,[1] it was only possible to obtain commercial samples that were distinguished by shade designation and to try them for different purposes. The only reason this investigation led to fairly

[1]Com. on Bact. Technic (1922). See References, p. 42.

definite conclusions was that the most satisfactory results were obtained with crystal violet which happens to be the one member of this series of dyes where the trade designation represents a distinct dye compound. The information is still indefinite, however, in regard to one of the methyl violets which has been found more satisfactory than crystal violet for certain purposes; for methyl violet, unlike crystal violet, is not a distinct chemical compound.

Theoretically, the way to investigate such a point as this would be to obtain a series of pure dye compounds representing the entire group under investigation, then to determine which of the pure dyes is most satisfactory for each of the various staining purposes for which the dye is desired. After learning this, it would ordinarily be possible to secure a commercial product consisting essentially of the desired dye compound, or if not, to develop a process for producing such a product. Such an investigation as this has seldom been made on account of the great difficulty in securing the pure compounds.

In one or two instances, however, it has been possible to carry on an investigation along the lines just indicated. Such a procedure has been made practicable by the cooperation of the Color Laboratory of the U. S. Dept. of Agriculture. The results of these investigations seem valuable enough to recommend the same method of attack in the study of other dyes and other staining procedures.

One of the investigations referred to is the study of eosin and its related dyes for use as bacterial stains by Conn and Holmes (1926). This particular investigation did not bring out anything of great practical value because the dyes found most satisfactory for the purpose, namely rose bengal, phloxine, and erythrosin, were the ones that had already been employed in the past. The work did, however, show so much difference between the closely related members of the group and such unexpected influence from changes in the chemical reaction that the value of such a method of investigation was brought out emphatically.

As a result, a similar method of investigation dealing with thionin and its derivatives was begun under the auspices of the Commission, the first part of which has been published by Haynes (1927). This investigation is not yet complete, but its results have already pointed out conclusions of practical value. This investigation, indeed, confirms the fact that pure methylene blue (as had already been brought out by previous investigation) has very little value as a nuclear stain for sections of fixed material, and also indicates that the higher members of the group, namely the ethyl derivatives, are equally poor in this respect. The investigation also confirms the

earlier conclusions that the principal reason for ripening a methylene blue solution or for preparing it with some alkali is to produce the lower homologs (azures, etc.). The recent investigation has gone further, however, in showing that almost without exception, each successive lower homolog from methylene blue down to a thionin shows better nuclear staining ability and more metachromasy than the higher compounds. Such an investigation as this suggests that it may be possible eventually to give up the use of methylene blue for many of its present staining procedures and to use instead whichever one of the lower homologs is especially adapted to the particular technic employed.

A similar investigation of acid fuchsin has been undertaken by certain members of the Commission which is published below (p. 50). The methods of producing acid fuchsin do not so readily yield pure dye compounds; but it has proved possible to obtain a sufficiently varied series to yield valuable results.

Needless to say, the groups of dyes just mentioned are not the only ones in which such an investigation as this can be made with the hope of obtaining profitable results. As stated above, the actual value of the different compounds of the methyl violet group is still unknown. A similar situation exists in regard to the safranins. Altho an investigation of the fluorescein group has been made as bacterial stains, the relative value of these dyes for most histological work is still unknown. There are various different methyl blues and anilin blues which are known to have different staining effects, altho the exact properties of the various compounds are entirely unknown. The list could be multiplied, and it will be a long time before accurate knowledge covering the whole field will be at hand. The subject is mentioned here in hopes that other investigators will be stimulated to take up the matter. It is hoped that anyone interested in carrying on such an investigation will communicate with the Commission about it. It cannot be promised that a series of pure dyes can be furnished to any investigator desiring the same, as the preparation of such a series often requires weeks or months of painstaking work; but it will help very much in planning the future investigations of the Commission if the interests of others along similar lines are known.

H. J. Conn

Thionin and Its Derivatives

As a result of recent work such as that of Holmes and French (1926) and Haynes (1927) more knowledge is now at hand as to the group of dyes embracing thionin and methylene blue than has ever

been available before. In the book, Biological Stains, the simple methylated derivatives of thionin discussed were merely methylene blue and methylene azure. Methylene azure was recognized as a synonym of azure I and was known to be ordinarily a mixture of azure A and azure B. The recent methods of MacNeal (1925), however, have shown how to obtain a fairly pure azure A; and Holmes and French (1926) give a method for preparing another compound which they call azure C.

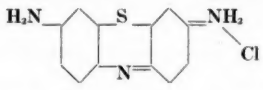
The adoption of these methods by stain manufacturers has put upon the market azures which are no longer the mixture formerly sold as methylene azure or azure I. They are much more nearly pure dye compounds than those which were previously available. The Commission has recently been accepting azure A prepared by MacNeal's method as methylene azure. It seems advisable in the future to discontinue this practice and to recognize azure A, azure B, and azure C as the separate dyes which they actually are. Accordingly, new definitions of these dyes are necessary and new specifications will shortly be drawn up. The following discussion of the properties of the dyes in question, altho it has all appeared in recent literature, seems advisable to clarify the situation and to show what steps the Commission proposes to take in the recognition of these dyes in the future.

THIONIN C. I. No. 920.

SYNONYM: LAUTH'S VIOLET

(Absorption maximum about 602)

This dye is ordinarily the chloride of diamino-diphenazthionium.



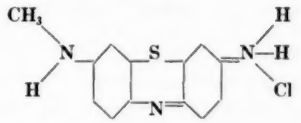
Altho recognized in the Colour Index, this product is no longer used as a textile dye, and immediately after the war there was some difficulty in securing it in the United States; it was apparently confused with an entirely different dye, thionin blue (C. I. No. 926). This trouble has been entirely overcome, and thionin is now readily available in good quality from the well established producers of biological stains in this country.

Thionin is violet in dilute solution, and has a very pronounced metachromatic effect, the colors in sections stained with it ranging from blue to reddish violet.

Azure C.

(Absorption maximum about 611)

This dye is mono-methyl thionin:



It is not a textile dye; and up to the present time has been manufactured only by Holmes and French, who prepared it by the oxidation of methylene blue. A small lot, prepared by them, is now on sale by the National Aniline and Chemical Co. This dye, like the other azures described below, has up to the present been prepared only by the oxidation of methylene blue. If they prove sufficiently valuable, a process of manufacture by direct synthesis will undoubtedly be developed. A product thus manufactured would probably be cheaper as well as purer than the present supply.

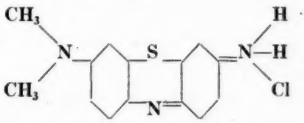
Azure C is recommended by French (1926) with orange II and eosin as a tissue stain. Haynes (1926) finds it possible to obtain equally good results, however, with azure I (i. e. azure A) if the technic is slightly modified. Later (1927) she reports excellent staining with it; but it is still uncertain whether it gives results sufficiently different from thionin on the one hand and azure A on the other to be of decided histological value. It is planned to allow the present azure C to be sold under the Commission certification however; for there is no question as to its giving good results if a suitable technic is employed in its use.

Azure A.

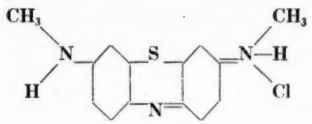
Synonym: Methylene Azure, Azure I.

(Absorption maximum about 638).

This is now recognized as asymmetrical dimethyl thionin:



It is not a textile dye; but is probably present in much of the methylene blue on the market. It must be distinguished from the isomeric symmetrical compound:



which has never been given a special name. Ever since first recognized by Kehrmann (1906) the impression has been current that this latter dye has no staining value and is of a distinctly different category from the azures. It is difficult to tell how this impression has arisen, and it possibly may have been due to work with an impure dye. Haynes (1927) finds both isomers to have almost identical staining properties.

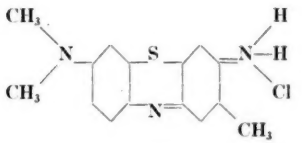
There is no mistaking the value of azure A; it is in fact regarded by MacNeal (1925) as the most important nuclear staining constituent of polychrome methylene blue. It is called for in all recently proposed formulae for the tetrachrome blood stain. It has been certified by the Commission for some time under the name of methylene azure; and it is proposed in the future to certify it under the more definite name, azure A.

TOLUIDINE BLUE O. C. I. No. 925.

SYNONYM: METHYLENE BLUE O.

(Absorption maximum about 635).

This dye theoretically does not belong with the others discussed here, as it is not a simple methylated derivative of thionin, having one methyl group in such a position as to give one-half the molecule toluidine structure:



It is included here, however, because of its similarity to azure A. It differs from the latter only in the presence of the extra methyl group attached directly to the benzene ring, and should theoretically have very similar properties.

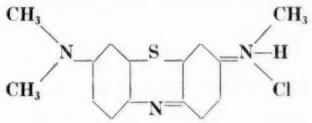
Toluidine blue has well recognized staining value, being a constituent of Pappenheim's panchrome stain and of the Albert diphtheria stain. It is still undertermined whether it can be used in place of azure A: but it has already been substituted for thionin in certain procedures. If it proves a generally satisfactory substitute,

its value will lie in the fact that it is already a commercial product, synthetically prepared.

Azure B.

(Absorption maximum about 652).

Azure B is the tri-methyl derivative of thionin:



Like azure A, this compound was first recognized by Kehrmann (1906) as a constituent of polychrome methylene blue. It can easily be prepared in impure form by oxidation of methylene blue, and is now available from the National Aniline and Chemical Co., if anyone desires to obtain it.

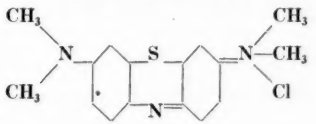
MacNeal (1925) states that its staining affects are much the same as can be obtained with a mixture of azure A and methylene blue, and that it therefore can be of little importance in polychrome methylene blue. Altho he adds that future study may change his opinion, his statement has generally been interpreted as meaning that azure B is not a satisfactory stain. Holmes and French (1926) also state that this dye has little staining value; but base their conclusions upon work with an impure sample, the performance of which was so poor as not apparently to justify further purification. Miss Haynes' recent work (1927) in this laboratory on sections of fixed tissue, with this sample of azure B, agrees partially with this, altho she finds the dye, if used in buffered solution, rather better than fairly pure methylene blue in fresh solution. There are indications that a purer sample of azure B may be still better. The Commission is not yet planning, however, to put this dye on the certification basis.

Methylene Blue C. I. No. 922.

Synonym: Swiss Blue

(Absorption maximum, if pure, about 667)

Methylene blue is theoretically tetra-methyl thionin:



The dye is so readily oxidized, however, that it is practically impossible to obtain in pure form, the presence of some of the azures or of methylene violet being almost universal. The commercial methylene blue, moreover, is ordinarily a zinc salt; the so-called medicinal grade is, however, free from zinc and much more satisfactory as a stain.

Methylene blue has long been recognized as an important nuclear stain. It is notable, however, that all the staining solutions specially recommended for use on sections of fixed tissue call for some ripening or for the addition of alkali. Such solutions are bound to contain one or more of the azures. There are indications, in fact, that pure methylene blue is a relatively poor histological stain.

The chief type of methylene blue which the Commission has recognized in the past has been the medicinal grade. This policy will be continued in the future, for it seems to be generally satisfactory for histological work, for blood stains, and apparently for vital staining. No effort will be made, however, to insist on a tetra-methyl thionin absolutely free from oxidation products.

H. J. Conn

The Microscopist's Need For Data on Dye Solubilities

In the preparation of saturated solutions of stains it is customary to allow the solvent and an undetermined excess of dye to stand in contact for an indefinite period with occasional agitation. In employing such solutions in staining it is customary to replenish them from time to time with further additions of solvent.

The Commission on Standardization of Biological Stains has pointed out,[1] that variation in the salt-content of stains will result in variation in the dye-content of their saturated solutions and has recommended that staining solutions be prepared, in so far as may be possible, on the basis of actual dye-content.

Brief reference may be made to several aspects of the matter which were not touched upon in the original discussion of the question.

The inorganic salts present in stains are only very slightly soluble in 95% alcohol but are readily soluble in water. The presence of appreciable amounts of salts in water decreases the solubility of dyes in a material degree. The effect of variation in the salt-content of stains upon the character of their saturated solutions depends, accordingly, upon the solvent employed. With aqueous solutions it may be very considerable. With alcoholic solutions it will be less decided, but may still be appreciable.

[1]Biological Stains, p. 24.

When saturated aqueous solutions of stains are prepared in the manner described, the dye-content of the solution depends upon the salt-content of the solution. The latter depends in turn upon the salt-content of the stain and also upon the ratio of stain and solvent employed. The dye concentration of the solution decreases with increasing salt-content in the dye and also with increase in the excess of dye used. Consistent results cannot be obtained with the same dye sample unless a constant ratio of stain and solvent is employed.

When such a saturated aqueous solution is employed in the manner outlined (i. e., with the occasional addition of fresh solvent) it is evident that its salt-content will be decreased in a material degree each time the solution is replenished. Each successive addition of solvent will be attended by an appreciable increase in the solubility of the dye, and the dye-content of the solution will increase progressively as long as the practice is continued.

A second objection to the use of saturated solutions of stains is equally valid irrespective of the solvent employed. Such dyes as fuchsin and safranin ordinarily contain approximately equal proportions of two homologs, and the character of other important stains is even more heterogeneous. The solubilities of the various components of any stain in any solvent invariably differ in some degree. In the freshly prepared saturated solutions of such stains the more soluble components of the dye will predominate. With continued use and repeated replenishing with solvent the essential character of the solution will become altered and eventually the less soluble components of the dye will predominate.

Saturated solutions of stains, in brief, may be of indefinite dye-content and may be subject to appreciable alteration in use, in both the content and the essential chemical character of the coloring matters present.

The preparation of staining solutions on the basis of actual dye-content, as advocated by the Commission, is a decided improvement in technic which cannot fail to yield more consistent results in general.

Solutions of definite dye-content can be prepared only when the dye-content of the stain is stated on the label of its container. The majority of stains certified by the Commission now contain such statements, which are supplied by the Color Laboratory of the Bureau of Chemistry. The analysis of dyes presents numerous difficulties, and the development of satisfactory and reliable methods for determining dye-content may necessitate extensive research in individual instances. Such investigations are being carried out as oppor-

tunity is afforded, and it is anticipated that all certified stains will eventually be of accurately determined dye strength.

Even when the actual dye content of all the stains on the market is known it will not be possible immediately to put staining formulae on a dye-content basis. Staining technic in the past has been grounded to a considerable measure upon the employment of saturated solutions; hence to interpret the old formulae into terms of actual dye content will very often necessitate a knowledge of the approximate dye content of saturated solutions. Probably no suggestion has been brought to the attention of the Commission more often than that the next edition of Biological Stains should include data as to the solubility of the various dyes listed. This information has been requested not only to help put old formulae on a dye-content basis, but also in order to assist the laboratory technician in employing the saturated solutions of the old formulae without too great waste of material.

These suggestions have been made without appreciation of the fact that such data are almost lacking in the literature. Solubility data in regard to dyes are, in fact, quite hard to obtain, partly because of the influence of impurities present upon the amount going into solution, and partly because of the difficulty of telling whether or not a solution has become completely saturated with the dye in question.

The cooperation of the Color Laboratory has now also been secured in obtaining this information. It is believed that the determination of the solubilities of stains in the solvents with which they are usually employed will be of sufficient value to the users of biological stains to justify an extensive investigation. The Color Laboratory will appreciate suggestions respecting the solvents which should be investigated both in general and also in specific instances.

The following paper, dealing with the data available in the literature, is the first of the series planned. It is hoped that a second paper, giving original data, will follow in an early issue of this JOURNAL; and that by the time a new edition of Biological Stains is called for the work will be sufficiently complete to furnish valuable data for insertion in that book.

W. C. HOLMES

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STAIN SOLUBILITIES

PART 1. DATA IN THE LITERATURE

W. C. Holmes

Contribution No. 130 from Color Laboratory, Bureau of Chemistry, Washington, D. C.

Summary.—**The rather meager data found in the literature concerning the solubilities of the dyes used as biological stains is reviewed. Solubility data have been found concerning the following dyes: picric acid, martius yellow, crystal ponceau, methyl orange, tropaeolin O, orange II, Bismarck brown, Congo red, auramine, malachite green, fuchsin, methyl violet, gentian violet, crystal violet, methyl green, diphenylamine blue, aurin, corallin, phenolphthalein, fluorescein, eosin Y, iodo-eosin, methylene blue, alizarin, indigo carmine, and carmine. Much of this information is of questionable reliability. The writer is investigating the matter and his original data are to appear in subsequent papers.**

Accurate solubility determinations on dyes involve numerous serious difficulties. It is probably for that reason that comparatively little information is available in the literature concerning the solubilities of dyes

The writer has, however, made a search thru the literature for solubility data on dyes; and has listed below such information as appears to be of interest to the users of biological stains.

Picric acid (C. I. No. 7):

100 gm. water at 20° dissolve 1.11 gm. (Dolinsky, 1905)
dissolve 1.22 gm. (Findlay, 1902)
dissolve 1.225 gm. (Marchand, 1847)
dissolve 1.45 gm. (Sisley, 1902b)

100 gm. water at 30° dissolve 1.40 gm. (Dolinsky, 1905)
dissolve 1.55 gm. (Findlay, 1902)
dissolve 1.525 gm. (Karplus, 1912)

100 gm. of a saturated solution in absolute alcohol contain
5.53 gm. at 12.3° (Behrend, 1892)
5.92 gm. at 14.8° (Id.)

100 gm. alcohol at 22° dissolve 8.37 gm. (Timofeiew, 1894)

100 gm. water at 25° dissolve 4.247 gm. of sodium salt. (Fisher and Miloszewski, 1910)

Martius yellow (C. I. No. 9):

Sodium salt dissolves in 35 parts of water. (Colour Index, p. 2).

Calcium salt dissolves in 265 parts of water. (Id.)
Ammonium salt is also soluble in alcohol. (Id.)

Crystal ponceau (C. I. No. 89):
A saturated aqueous solution at 17° contains 30.7 gm. per liter. (Pelet-Jolivet, 1910).
By warming to 55° and cooling to 20° a saturated solution was obtained at the latter temperature which contained 38.9 gm. per liter. (Id.)
1 liter of water at 23° dissolves 55.7 gm. of the sodium salt (+ 7 H_2O). (Sisley. 1902).
1 liter of water dissolves 209.6 gm. of color acid. (Id.)
1 liter of 5% H_2SO_4 dissolves 41.3 gm. of the color acid. (Id.)

Methyl orange (C. I. No. 142):
100 gm. water at 20–25° dissolve 0.02 gm. of methyl-orange (sodium salt). (Dehn, 1907b).
1 liter of water at ordinary temperature dissolves 0.055 to 0.225 gm. of helianthine (color acid). (Dehn, 1907a).
1 liter of alcohol dissolves 0.1 gm. (Id.)

Tropaeolin O (C. I. No. 148):
1 liter of water at 23° dissolves 4.22 gm. of dye (sodium salt + 2½ H_2O). (Sisley, 1902).
1 liter of water at 100° dissolves 200 gm. (Id.)
1 liter of water at 19.5° dissolves 2 gm. of color acid. (Id.)
1 liter of 1% HCl dissolves 0.06 gm. (Id).

Orange II (C. I. No. 151):
1 liter of water at 19° dissolves 41.16 gm. of dye (sodium salt + 5 H_2O). (Sisley, 1902a).
1 liter of water containing 1% salt dissolves 0.38 gm. of dye. (Id.)
1 liter of water at 19° dissolves 174.4 gm. of color acid. (Id.)
1 liter of 5% HCl dissolves 0.27 gm. of color acid. (Id.)

Bismarck brown (C. I. No. 331 or 332):
100 gm. water at 20–25° dissolve 8.5 gm. (Dehn, 1907b).

Congo red (C. I. No. 370):
100 gm. water at 20–25° dissolve 11.6 gm. (Dehn, 1907b).

Auramine (C. I. No. 655):
100 c.c. 96% alcohol at 20° dissolve 7.0 gm. of dye base (Finckh and Schwimmer, 1894).
100 c.c. ether dissolve 2.31 gm. (Id.)

Malachite green (C. I. No. 657):
Water dissolves 4% of dye. (Brunner, 1887).
Alcohol dissolves 5% of dye. (Id.)

Fuchsin (C. I. No. 677):
1 liter of a saturated aqueous solution
at 18° contains 1.875 gm. (Pelet-Jolivet, 1910)
at 44.5° contains 8.2 gm. (Id.)
at 67° contains 11.25 gm. After cooling to 18° for 12 hours this same solution contains 2.685 gm. (Id.)
Water dissolves 0.3%. (Brunner, 1887).
Alcohol dissolves 10%. (Id.)
100 gm. water at 20–25° dissolve 0.03 gm. rosanilin (apparently referring to the dye base). (Dehn, 1907b).
100 gm. water at 9° dissolve 0.241 gm. (Rosenstiehl, 1876).
100 gm. water at 6° dissolve 0.184 gm. (Id.)

Methyl violet (C. I. No. 680):
Water dissolves 2.0%. (Brunner, 1887).
Alcohol dissolves 1.5%. (Id.)

Gentian violet:
Water dissolves 1.5%. (Brunner, 1887).
Alcohol dissolves 3.0%. (Id.)

Crystal violet (C. I. No. 681):
A saturated aqueous solution at 17° contains 7.5 gm. per liter. (Pelet-Jolivet, 1910).
By warming to 55° and cooling to 20° a solution was obtained containing 20.7 gm. per liter. (Id.)
The iodide is much less soluble than the chloride. One liter of water dissolves only 0.2 gm. of the iodide. (Rosenstiehl, 1896).

Methyl green (C. I. No. 684):
Water dissolves 7%. (Brunner, 1887).
Alcohol dissolves 0.25%. (Id.)

Diphenylamine blue (C. I. No. 688):
100 gm. saturated solution in methyl alcohol at 23° contain 0.385 gm. (von Szathmary de Szachmar, 1910).
100 gm. saturated solution in ethyl alcohol at 23° contain 0.230 gm. (Id.)

100 gm. saturated solution in acetone at 23° contain 0.177 gm. (Id.)
100 gm. saturated solution in anilin at 20° contain 0.395 gm. (Id.)
The corresponding data for triphenyl pararosanilin hydrochloride are given as: Methyl alcohol 0.447 gm. at 23.5°; ethyl alcohol 0.285 gm. at 23°; acetone, 0.19 gm. at 21°; anilin 0.518 at 22°.

Aurin (C. I. No. 724):
Alcohol dissolves 40%. (Brunner, 1887).
dissolves 50%. (Cohn, 1907).
100 gm. water dissolve 0.12 gm. at 20–25.° (Dehn, 1907b).

Corallin (C. I. No. 726):
Water dissolves 2%. (Brunner, 1887).
Alcohol dissolves 0.5%. (Id.)

Phenolphthalein (C. I. No. 764):
100 gm. water at 20° dissolve 0.0175 gm. (Acree and Slagle 1909).
100 gm. water at 20–25° dissolve 0.04 gm. (Dehn, 1907b).
Soluble in 10 parts alcohol. (Cohn, 1907).

Fluorescein (C. I. No. 766):
100 gm. water at 20–25° dissolve 0.005 gm. of color acid. (Dehn, 1907b).

Eosin Y (C. I. No. 768):
100 parts water (cold) dissolve 40 parts of the potassium salt. (Depiere, 1875).
1 part of esoin dissolves in 11 parts of "Weingeist." (Id.)
Water dissolves 2%. (Brunner, 1887).
Alcohol dissolves 1%. (Id.)

Iodo-eosin (C. I. No. 773?):
100 gm. water at 20–25° dissolve 90 gm. (Dehn, 1907b).

Methylene blue (C. I. No. 922):
1 liter of a saturated aqueous solution
at 18° contains 11.3 gm. (Pelet-Jolivet, 1910).
at 44.5° contains 95.5 gm. (Id.)
100 gm. water at 20–25° dissolve 4.36 gm. (Dehn, 1907b).
100 gm. water are stated by an unknown investigator to dissolve 5.6 gm. at 20° and 8.1 gm. at 25°.

Alizarin (C. I. No. 1027):

1 liter of a saturated aqueous solution at 25° contains 0.00006 gm. (Hüttig, 1914).

100 gm. water at 20–25° dissolve 0.04 gm. (Dehn. 1907b).

100 parts of water at 100° dissolve 0.034 parts of dye. (Schützenberger and Plessy, 1856).

Indigo carmine (C. I. No. 1177).

The potassium salt dissolves in 140 parts of cold water. (Beilstein).

The sodium salt is soluble in from 140 to 150 parts of water. (Cohn, 1907).

Carmine (C. I. No. 1239):

100 gm. water at 20–25° dissolve 0.13 gm. (Dehn, 1907b).

Many of these data appear to be of questionable reliability. In various instances the identity of the materials seems uncertain, their purity doubtful, and the methods by which they were investigated crude.

An investigation of the solubilities of biological stains in water and in 95% ethyl alcohol is now being undertaken at the Color Laboratory of the Bureau of Chemistry. The data obtained will appear in subsequent paper.

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ACID FUCHSIN AS A STAIN—A REFINEMENT IN MANUFACTURE*

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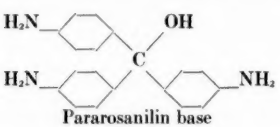
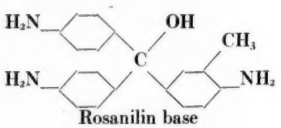
ABSTRACT.—**From the study of 38 samples of acid fuchsin prepared from several types of basic fuchsin and under varying conditions it is found that rosanilin sulfonated between 80° and 85°C. gives the best results in the Van Gieson staining technic. Staining tests also show that a satisfactory acid fuchsin will give the best results when employed with picric acid in the ratio of 1 part of the 1 per cent aqueous acid fuchsin to 20 parts of the aqueous picric acid. Details for the preparation and use of acid fuchsin are given.**

While the Van Gieson method of staining connective tissue has long been considered excellent, an outstanding problem in its proper consummation has been a satisfactory acid fuchsin. The majority of samples have wholly failed to give the proper staining reaction when employed in conjunction with picric acid in the customary method and many that have resulted in satisfactory stains have been notoriously fugitive. Fading is a common fault with many Van Gieson preparations.

Since all of the acid fuchsins on the market are sulfonation products of ordinary basic fuchsin (magenta), which is a mixture of at least two homologs, it was thought probable that the presence or absence of one of these was the determining factor in the variation of the commercial products. To determine whether or not this was the case, samples of the two pure homologs which ordinarily occur in basic fuchsin were obtained, namely pararosanilin and rosanilin (homorosanilin). They were furnished by the manufacturers as the dye bases. The formulae for these two compounds are:



Pararosanilin base



Rosanilin base

A third homolog also was included, the dye known as new fuchsin. This was furnished by the manufacturer as the chloride, which has the formula:

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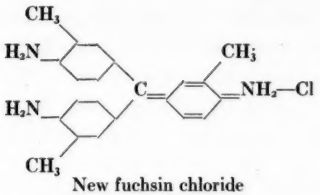
*Application and staining tests were made by the Army Medical Museum, 7th and B Sts., S. W., Washington, D. C.



New fuchsin chloride

From the two dye bases the chlorides were prepared by solution in dilute hydrochloric acid, using one molecule of the HCl for each molecule of the dye base. The chloride was recovered by evaporating the solution to dryness. It is well to note at this point that excess hydrochloric acid in this step has a very bad effect. If too much of the acid is used, the solid chloride has a dull red-violet color and its sulfonation product is not satisfactory. The dry, solid chloride should have a bright green color and a bronzy luster. Such material almost invariably gave better results on sulfonation.

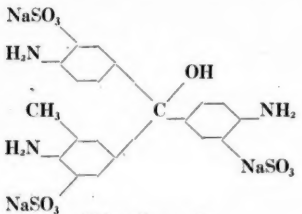
The method of sulfonation employed was essentially the same as that outlined in U. S. Patent 250,247, except that this patent is very vague in the matter of temperature. It specifies "ordinary" or "moderate" temperatures. The temperature in these experiments was varied over a wide range, from room temperature to 175°C. The strength of the fuming sulfuric acid also was varied, but this seemed to have little effect, provided there was some excess of free SO_3 in the reaction mixture after all the dye to be sulfonated had been dissolved. That was found to be essential. When the NaOH test indicated incomplete sulfonation after all the dye had been dissolved, further heating had no effect; but the addition of sufficient fuming sulfuric acid to yield an excess of free SO_3 gave immediate and complete sulfonation.

Temperature seems to be the most important factor. When temperatures above 110°C. were used, the product was practically useless. While a sample of rosanilin sulfonated at 100°C. was quite fair in staining results, the same rosanilin sulfonated at 125°C. afforded a dirty brownish red stain, and that sulfonated at 150°C. resulted in a dark green connective tissue stain. Neither of the latter stains was of sufficient strength or selectivity to be of material interest. Similar results appertain to pararosanilin and new fuchsin, except that the latter showed less promise as a stain at all temperatures.

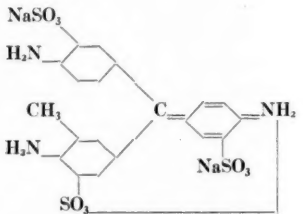
At room temperature results also were unsatisfactory. Between 85°C. and 110°C. fair results were obtained. Excellent products resulted, however, when the temperature was allowed to rise only to 80° to 85°C. and kept within that range during the entire reaction.

Using material sulfonated under the conditions which gave the best sulfonation products, a comparison was made between the three homologous dyes. Under these optimum conditions of acid concentration and temperature, it was found that pararosanilin gave a product which was very fair, but off in shade, being too dark and lacking selectivity; while the new fuchsin product was too diffuse in stain action. A sulfonated rosanilin, on the other hand, displayed excellent qualities, both as to shade and selectivity, in combination with picric acid, and was much more satisfactory than products obtained from either pararosanilin or new fuchsin. Further investigation with the object of working out the minor details of the preparation was confined to rosanilin.

Excellent results were obtained by sulfonating both the rosanilin base as supplied by the manufacturer and the rosanilin chloride, with the results rather favoring the use of the dye base. The procedure which gave the best results is given in detail below. Special attention is called to the matter of acidification after the precipitation and removal of $CaCO_3$. The acid fuchsin at that point is in solution as the sodium salt, principally the trisodium salt, which is colorless or pale yellow. For the purpose for which this product is intended, it is best to convert as much of it as possible to the highly colored (magenta red) di-sodium salt, thus



Tri-sodium salt
(colorless)



Di-sodium salt
(red)

When all of the tri-sodium salt has been converted to the di-sodium salt (which is neutral) further addition of hydrochloric acid gives an acid reaction, because of the presence of free HCl in the solution or because of the presence of the mono-sodium salt of the dye which is acid. Addition of HCl must be stopped at exactly the correct point, which is indicated by a faint acid reaction to congo red paper. Excess of hydrochloric acid at this stage will ruin the product, especially if it is recovered, as the procedure below specifies, by evaporation to dryness. In drying a temperature of 100°C. should not be exceeded in any case.

The change of the tri-sodium to the di-sodium salt of acid fuchsin is the mechanism of the Andrade indicator employed in bacteriological technic. The addition of alkali to the acid fuchsin results in the formation of the colorless compound, while acid formed by bacterial metabolism accomplishes the transformation back to the red, di-sodium compound. This reaction is much less sensitive than that of tissue staining and any acid fuchsin which meets the requirements as a stain will easily meet the needs of the bacteriologist as an Andrade indicator.

Preparation

The reaction was carried out in a 300 c.c. tall-form beaker equipped with an efficient mechanical stirrer, a thermometer and a pan to serve as a container for the paraffin heating bath. The apparatus was placed in a hood to carry off the SO_3 and HCl fumes. In the beaker were placed 50 grams of cold fuming sulfuric acid (23.2% free SO_3) and, with continuous stirring, 10 grams of finely ground rosanilin base (or rosanilin chloride) were added little by little. The temperature rose rapidly to 80°C., and the rate of addition was then regulated so that the temperature remained between 80° and 85°C. With one batch of material, lumps formed; but the addition was not interrupted, and the lumps eventually dissolved. When all the material had been added the temperature began to fall, and the paraffin, which had been previously heated on the steam bath, was poured into the bath surrounding the beaker. By gently heating this bath, the temperature was kept at 80° to 85°C. and the vigorous stirring continued for one hour. When sulfonation was complete a drop of the reaction mixture in an excess of cold dilute NaOH yielded a clear, colorless or faintly yellow solution. Decolorization was practically instantaneous. At the end of the hour the reaction mixture was cooled and poured into 500 c.c. of cold water in a 2000 c.c. beaker. This solution was heated to boiling and neutralized with solid $CaCO_3$. The $CaSO_4$ was removed by filtering the hot solution with suction and washed with 500 c.c. of hot H_2O. The volume of the solution was made just sufficiently large for convenience in neutralizing and filtering. The larger the volume, the more inorganic salts the final product contained. The combined filtrate and washings were then heated to boiling and the calcium precipitated by the addition of $NaHCO_3$. About 12 to 14 grams were required. A test was made for the complete precipitation of the calcium by filtering off a small portion of the solution, heating the filtrate to boiling and adding a few drops of a hot solution of $NaHCO_3$ in H_2O. When the test showed complete precipitation, the solution was allowed to cool and to stand over night.

The $CaCO_3$ was then filtered off and washed with 200 to 300 c.c. of cold H_2O. The combined filtrate and washings were then acidified by adding dilute HCl (6N.) 0.5 c.c. at a time until a faint acid reaction to Congo red paper was obtained. The amount of 6 N. HCl required varied from 10 to 20 c.c. The solution was then filtered cold into a porcelain dish and evaporated to dryness on the steam bath. When thoroly dry the material became very brittle and separated readily from the dish. It was ground in a mortar. The best products when dry had a bright green color and a beautiful bronzy luster. The poorer products were characterized by a reddish violet color when dry and no luster whatever.

Staining Technic.

In making comparisons of samples of acid fuchsin, the following technic was employed:

1. Stain in Weigert iron hematoxylin for 5–10 minutes.
2. Wash in water.
3. Stain in the following Van Gieson mixture 5 min.

Saturated Aq. picric acid	20 parts
1% Aq. acid fuchsin	1 part.

4. Dehydrate and differentiate in 95% alcohol.
5. Absolute alcohol, 3 min.
6. Cedarwood oil, 1 min.
7. Xylol.
8. Mount in xylol balsam or damar.

Study of the Van Gieson mixture has shown that the ratio of 1 part of the 1% aqueous acid fuchsin to 20 parts of the saturated aqueous picric acid is best. Other ratios, with a satisfactory acid fuchsin, gave inferior results. Too small a quantity of acid fuchsin results in a weak red stain, while increasing the proportions of fuchsin results in an overpowering red stain, impossible to differentiate. Furthermore, it is noted that a good sample of acid fuchsin will also afford a satisfactory nerve stain in the 1 to 20 mixture.

For consistent and clean cut results, the Weigert iron hematoxylin has proven superior. Other nuclear stains have been satisfactory at times but not so consistently. The Weigert stain affords a sharp nuclear picture with little diffusion. Any diffusion of the nuclear stain is fatal to a clean cut acid-fuchsin-picric-acid stain, as an underlying color will detract greatly from the normally bright yellow of the picric acid.

Tissues from various sources, both formol and Zenker fixation, were employed as test objects. Little difference is noted with regard

to Zenker fixed material from formol preparations in the application of the Van Gieson stain.

Water should be avoided following the use of the Van Gieson mixture. Water will result in more or less extraction of the dye as well as a general dulling of the stain picture. It is found to be better for a clean cut result to carry the tissue directly from the acid-fuchsin-picric-acid to 95% alcohol. Differentiation is accomplished quickly, and with a proper dye over-differentiation appears to be impossible.

With regard to color-fastness, it is noted that certain dyes appear to be quite permanent if proper staining has been accomplished. From the standpoint of appearance, 30 seconds to a minute is often sufficient to secure a good Van Gieson stain; but, such preparations are much more likely to fade than those which have been stained according to the above schedule. After the Weigert iron hematoxylin for nuclear staining, it is easy to secure good differentiation and clean results with an even longer staining period in the Van Gieson mixture.

The following table gives the staining results obtained with acid fuchsins obtained by the sulfonation of the various basic fuchsins at different temperatures:

Lot No.	Dye	Temp. of Sulfonation	Staining Result
12	Rosanilin	Room Temp.	Poor
13	Pararosanilin	Room Temp.	Not good.
14	New fuchsin	Room Temp.	Poor.
37	Rosanilin	80–85°C.	Excellent.
21	Pararosanilin	80–85°C.	Very good.
24	New fuchsin	80–85°C.	Good.
11	Rosanilin	100°C.	Poor.
20	Pararosanilin	100–105°C.	Fair.
23	New fuchsin	100–105°C.	Fair.
4	Rosanilin	125°C.	Not good.
19	Pararosanilin	120–125°C.	Poor.
22	New fuchsin	120–125°C.	Poor.
7	Rosanilin	150°C.	Not good.
5	Pararosanilin	150°C.	Not good.
6	New fuchsin	150°C.	Not good.
8	Pararosanilin	175°C.	Not good.

STAIN TECHNIC FOR NISSL'S GRANULES FOLLOWING ALCOHOL-FORMOL FIXATION

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ABSTRACT.—1. Nerve tissue is fixed 2–4 hrs in a 5% solution of strong formalin in commerical 95% alcohol.

2. If dehydration is perfect, either chloroform or xylol may be used as a clearing agent.

3. A slow method of paraffin infiltration is advisable.

4. Sections should be cut 10–12 microns in thickness.

5. Coplin staining jars should be annealed by placing them on a rack in a pan of cold water, bringing the water to the boiling point, and allowing the jars to stand in boiling water for twenty minutes.

6. One per cent aqueous solutions of either methylene blue or Grübler's Neutral Roth are used as specific stains for Nissl's granules.

7. These stains are heated to boiling in a beaker, the slides are placed in the Coplin jars which are partially submerged in boiling water, and the hot stain poured into the jars. The flame beneath the water bath is turned down and the slides left for 20 minutes.

8. The excess of primary stain is washed off in 25% and 50% alcohol and the slides passed rapidly thru the alcohol series to absolute alcohol, and finally to xylol.

9. When counterstaining is desired, nigrosin in 1% aqueous solution, methyl orange, saturated solution in 50% alcohol, or, a 0.5% solution of eosin in 50% alcohol are recommended. These stains are used cold, and the slides are merely dipped in them after the excess of primary stain has been washed out in 25% and 50% alcohol.

10. If a cold primary stain is desired, a saturated solution of thionin in distilled water, acidified with 1% carbolic acid, will prove specific for the Nissl substance. Sections should be stained 5–10 minutes in thionin, then passed rapidly thru to absolute alcohol, and xylol. The same counterstains may be used as in the hot method.

11. Sections prepared by the hot method show little tendency to fade after ten years use.

12. Excepting neutral red, all the stains used in this technic are carried by the National Aniline and Chemical Company and are satisfactory. Coleman and Bell neutral red may be substituted for Grübler's Neutral Roth with good results.

A common criticism of the Nissl method for staining nerve cells, is the fact that such preparations show a strong tendency to fade

within a comparatively short time. The modification of the Nissl method which is herein presented, is recommended to check this tendency to fade and to insure fairly uniform results in the hands of even a beginner in technic. Sections prepared by this modified Nissl method ten years ago have been in constant use in the class room, yet the colors are as brilliant to-day as on the day when the slides were finished.

Satisfactory results from this technic, will depend largely upon the treatment of the tissue previous to staining. Therefore, a few suggestions on the material selected and the handling of such material up to the time of staining, are permissible.

Material

For those who have had little experience in neurological technic, sections of spinal cord will prove excellent material. Among the mammals, the spinal cord of the pig is very satisfactory since it is easy to secure and the motor cells in the anterior horns of the gray matter, are very large. It will greatly facilitate the penetration of fixing, dehydrating, and clearing fluids, if the cord be cut transversally into segments about one centimeter in length, and these segments in turn be cut thru the antero-posterior medial plane.

Fixation

One of the most valuable fixatives for nerve tissue, is a 5% solution of strong formalin in commercial 95% alcohol. Half a dozen pieces of tissue placed in 100. c.c. of this fluid, will be thoroly fixed in two or three hours. Absolute alcohol and formalin in the same proportions may be used if desired, but the method is more costly and there seems to be little advantage in its use.

Dehydration and Clearing

After fixation the tissue should be left in 95% alcohol for at least twenty-four hours, to harden. Dehydration in absolute alcohol should extend over a minimum period of three hours, and the alcohol changed at least once during this time. Either chloroform or xylol may be chosen as a clearing agent, with the minimum time limit set at three hours, and taking care to change the clearing agent at least once.

Infiltration

It is advisable to use a slow method of infiltration, gradually adding soft paraffin to the clearing fluid which is kept at a temperature of about 34° centigrade, until the saturation point is reached. The

tissue may now be submerged in melted hard paraffin for two hours, changing the paraffin twice. It is to be noted that with the exception of infiltration with hard paraffin, the time limits of any of the steps mentioned heretofore may be extended an hour or two without detrimental effects on the tissue.

Sectioning

For ordinary purposes of demonstration there is no advantage in cutting nerve sections less than 10 or 12 microns in thickness. If the albumin is of good quality and spread in an even film over a perfectly clean slide, sections will dry out in a drying oven within two days, and none will be lost in the staining procedure to follow.

Staining (Hot method).

As a preliminary to the use of hot stains, it is advisable first to submit Coplin staining jars to an annealing process. If the glass is of fair quality this is easily done in the following way. The jars are placed on a rack in a pan, cold water is poured into the pan until each jar is half submerged, and the water is heated to boiling for twenty minutes.

Two primary stains may be used with equally fine results, 1% aqueous solutions of methylene blue or Grüblers Neutral Roth. The chosen stain is heated to boiling in a beaker. The slides are placed in the Coplin jars which are already partially submerged in boiling water, and the hot stain is next poured into the staining jars. The flame beneath the water bath should be turned down, and the slides left for twenty minutes in the hot stain. When ready for washing, dehydration, and mounting, each slide should be handled separately in the following way: It is first dipped for 15 seconds in 25% alcohol, then successively in 50%, 75% and 85% alcohol. At this stage it is well to examine it under the microscope to determine how fast the stain has come out. After a trial or two however, one learns that a faint pink or a pale blue tint in the white matter indicates that Nissl's granules are deeply stained with the dye. From 85% alcohol the slide is transferred to 95% alcohol and left for two minutes, then passed successively to absolute alcohol and xylene. To insure perfect dehydration and clearing, it is well to use two jars of absolute alcohol and two of xylol.

Using this procedure, the neurons stand out in bold relief on a practically colorless background. When counterstaining is desired, four dyes are specially recommended,—nigrosin, methyl orange, eosin, or erythrosin. When the excess of primary stain has been

washed out in 25% and 50% alcohol, the section may be dipped for a moment in a 1% aqueous solution of nigrosin, rinsed in 25% alcohol, and passed rapidly up to 95% alcohol. It will be found that the white matter of the cord is stained a bluish-gray and if neutral red is used as the primary stain the contrast is striking. Following the same procedure as for nigrosin, a saturated solution of methyl orange in 50% alcohol gives a metallic yellow counterstain, while a 0.5% solution of eosin in 50% alcohol gives the characteristic red counterstain.

Staining (Cold method)

For a cold primary stain, thionin gives excellent results. Sections stained from 5 to 10 minutes in a saturated aqueous solution of thionin acidified 1% with carbolic acid, (King's acid thionin), are rinsed lightly in distilled water and passed rapidly thru the alcohol series to xylol. It is just as specific for the Nissl bodies as methylene blue, and stains a deep violet hue. After washing away the excess primary stain, the same counterstains mentioned above may be used with excellent results. A grayish-red counterstain may be obtained by exposing sections to a 1% solution of bordeaux red for 5 seconds, rinsing in water, then staining for 10 minutes in thionin.

The hot method of staining was originally worked out with Grübler products. As the German supply gradually gave out, attempts were made to find satisfactory American substitutes. It was soon discovered that the new methylene blue dispensed by the National Aniline and Chemical Company worked very well. Their dye listed as Erlich's neutral red, shows no specificity for the Nissl substance. Unsatisfactory results also followed the use of a neutral red which the Will Corporation handled a few years ago. The Coleman Bell product, however, works perfectly, altho the stain is much darker and less brilliant than the Grübler preparation.

Excepting neutral red, the stains carried by the National Aniline and Chemical Company will prove satisfactory for the technic described above.

OIL RED O PYRIDIN, A RAPID FAT STAIN

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In a brief communication in this Journal[1] on comparative study of fat stains by R. W. French, two commercial products—candle scarlet (Geigy) and oil red O (National Anilin) were stated to be superior to a special purified sudan III.

My experience with one of the fat stains, oil red O, confirms French's statement.

Oil red O, according to the manufacturer's statement, is an azo dye formed by diazotation of amino-azo-xylene and condensation with beta napthol. It is a dark red crystalline powder soluble in all common fat solvents and used technically in the preparation of wood stains.

A saturated solution of oil red O in 70% alcohol, acetone or acetone-alcohol, will stain fats and lipoids a brilliant deep orange color. Compared with sudan III and sudan IV (scarlet red), its staining power is more intensive and requires a shorter time for staining and seems to make more fat visible, especially the minute fat droplets.

In a comparative study with various fat solvents, it was found that oil red O, as well as sudan III and scarlet red, is more easily soluble in 70% watery solution of pyridin, than in 70% or 80% alcohol. The former solution requires only two to three minutes to stain, while an alcoholic solution to stain in the same intensity, requires ten to fifteen minutes. Hence the pyridin solution is more concentrated and requires less time for staining.

Herxheimer[2] advocated for the same purpose a two per cent solution of sodium hydroxide in 70% alcohol as a solvent for scarlet red. Scarlet red in an alcoholic solution of sodium hydroxide forms a sodium salt, which is more easily soluble than the free dye. If fat-containing sections are stained in this solution, the free scarlet red dye diffuses into the fat droplets and the sodium remains in alcohol.

According to Herxheimer, an alkalized, alcoholic solution of scarlet red does not cause swelling of the cell elements, but in my experience it does, and this makes counter-staining of the nuclei difficult.

Pyridin, which has a slight alkaline reaction, does not alter the cellular structures, and in a 70% solution will not dissolve fat or lipoids if used at room temperature. It is, therefore, preferable to an alcoholic solution of sodium hydroxide. The 70% Pyridin solution

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[1] R. W. French, Fat Stains, Stain Technology, 1, p. 79. 1926.
[2] G. W. Herxheimer, Ueber Fettfarbstoffe. Deutsche Med. Woch. 1901.

can also be used as a fluid for differentiation instead of 70% alcohol. A further advantage of pyridin as a solvent instead of alcohol is that it does not cause precipitation of the stain.

Oil red pyridin is especially valuable for the demonstration of the lipoid degeneration products in the central nervous system. If a neutral alcoholic solution of sudan III, or scarlet red is used, several hours staining is necessary. Oil red pyridin will stain the same from twenty to thirty minutes to a deep orange red color.

Formula for making the staining solution and method of staining:

About 3–5 grams of oil red O are added to 100 cc. of 70% watery solution of pyridin (70 cc. pyridin C. P. and 30 cc. distilled water); after an hour standing at room temperature with occasional shaking the solution is saturated and ready for use. The solution should be kept in a glass stoppered bottle, protected from light, and filtered before use. The staining dishes should be well covered to prevent undue evaporation of the pyridin.

Frozen sections of tissues, fixed in formalin, Mueller-formalin, or formalin-picric acid,[3] are immersed in 50% pyridin for about three to five minutes, and than transferred in pyridin oil red for three to five minutes. Sections from central nervous system should be stained from twenty to thirty minutes. The sections are then differentiated in a 50% pyridin for several minutes,[4] transferred in water and counter-stained with Delafields' hematoxylin for two or three minutes. If sections of the central nervous system are to be counterstained with hematoxylin, an acidified solution of Delafield's hematoxylin is recommended. Sixteen cubic centimeters of Delafield's hematoxylin are acidified with 2 cc. glacial acetic acid to prevent overstaining of the cytoplasm. The sections are mounted in gum acacia or laevulose syrup.

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[3]100 cc. of saturated water solution of picric acid and 5 cc. of 10% formalin. This solution is especially recommended for the central nervous system.

[4]Since oil red stains the myelin sheathes quite intensively, brain sections should be differentiated for at least half an hour with 50% Pyridin.

NOTES ON TECHNIC

MORDANTING WITH USED FLEMMING'S KILLING FLUID. Material fixed in Bouin's, Carnoy's, or Ohlmacher's solutions may be induced to stain with Flemming's triple stain (safranin, gentian violet, and orange G) by mordanting the sections on slides for twenty-four to seventy-two hours in either fresh or used Flemming's killing fluid. The safranin must have been proved satisfactory on Flemming-fixed material. Wash slides five minutes after mordanting.

This method comes to me from Dr. H. de Winiwarter.

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ANNOUNCEMENT

TYPE COLLECTION OF MICROSCOPICAL STAINED PREPARATIONS

The Army Medical Museum, at the request and with the cooperation of the Commission on Standardization of Biological Stains, is establishing a type collection of stained microscopical preparations. This collection is to contain preparations representative of various microscopical staining technics and will be available for exchange and consultation by those interested in the results obtainable with various procedures.

In effecting standardization of biological stains the experience of the Stain Commission has been that as the result of the personal equation there is a marked variation in the results obtained and also in the results desired. There are marked variations in personal taste in appraising the success of a staining technic, as well as many instances where there is a decided misconception of the proper operation of various dyes as staining agents.

Such difficulties can be partly prevented if preparations from many laboratories are available for comparison; hence the establishment of a type collection is proposed. It is not planned to attempt a standardization of staining results, or to influence technic toward results of any particular nature, but rather to enable one to decide through direct examination what is considered satisfactory by others and especially by the originator of a given stain. A type slide from this collection, moreover, will in many instances show the limitations of the procedure involved and whether it is suited to a given problem.

When possible, the preparations for this collection are to be supplied by the originators of the various staining technics; otherwise by laboratories recognized as being fully competent to employ a particular stain in a wholly satisfactory manner. It is also hoped that any one announcing a new staining method will file with the type collection a sufficient number of slides to demonstrate fully the different reactions to be expected with various types of material and methods of fixation.

To make this collection a success, it must be as comprehensive as possible, including a large variety of procedures, and embracing all fields of microscopic work, both zoological and botanical. The inclusion of both animal and plant preparations is specially desirable; as analogies exist between the two fields which may be studied to advantage.

A difficulty which will no doubt be encountered in the maintenance of the type collection, is the fact that many commonly employed stains fade rapidly. It is therefore requested that all preparations be submitted in duplicate which will permit the maintenance of one file under the best possible conditions to obviate fading. In the event of appreciable fading, the original laboratory supplying a specimen will be called upon for additional fresh preparations, or suitable fresh duplicates will be obtained from other recognized sources.

Contributors to the collection are asked to forward the following data with all specimens: material stained, including method of preparation and fixation; technic in detail; dyes employed, their source, lot or certification number, and any other special designation which may help in their subsequent identification. (Information of this type concerning a dye is essential to secure duplication of a procedure.) All data received will be forwarded with slides circulated.

Slides of the type collection will be available for circulation upon request, but are to be returned to the collection, post paid, after a reasonable time for inspection and comparison. Suitable mailing containers will be employed to enable safe and easy shipping.

It is hoped that microscopists will avail themselves of the collection, both by submitting specimens and by sending in requests for them after the collection is established. Those likely to have slides to contribute, or interested in the idea are invited to correspond directly with the Curator, Army Medical Museum, 7th and B Sts., S. W., Washington, D. C.